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(54) Title: USE OF 2'-SUBSTITUTED OLIGONUCLEOTIDES TO DOWN-REGULATING GENE EXPRESSION

(57) Abstract

Disclosed is a method of down-regulating the expression of a gene in an animal, wherein a pharmalogical formulation comprising an oligonucleotide complementary to the gene is orally administered to an animal. The oligonucleotide administered has non-phosphodiester internucleotide linkages and includes at least one 2'-substituted ribonucleotide, the oligonucleotide inhibiting the expression of a product of the gene, thereby down-regulating the expression of the gene.

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USE OF 2'-SUBSTITUTED OLIGONUCLEOTIDES TO DOWN-REGULATE GENE EXPRESSION

BACKGROUND OF THE INVENTION

The present invention relates to the control of gene expression. More particularly, this invention relates to the use of synthetic oligonucleotides to down-regulate the expression of a gene in an animal.

The potential for the development of an antisense oligonucleotide therapeutic approach was first suggested in three articles published in 1977 and 1978. Paterson et al. (*Proc. Natl. Acad. Sci. (USA)* (1977) **74**:4370-4374) discloses that cell-free translation of mRNA can be inhibited by the binding of an oligonucleotide complementary to the mRNA. Zamecnik et al. (*Proc. Natl. Acad. Sci. (USA)* (1978) **75**:280-284 and 285-288) discloses that a 13mer synthetic oligonucleotide that is complementary to a part of the Rous sarcoma virus (RSV) genome inhibits RSV replication in infected chicken fibroblasts and inhibits RSV-mediated transformation of primary chick fibroblasts into malignant sarcoma cells.

These early indications that synthetic oligonucleotides can be used to inhibit virus propagation and neoplasia have been followed by the use of synthetic oligonucleotides to inhibit a wide variety of viruses, such as HIV (see, e.g., U.S. Patent No. 4,806,463); influenza (see, e.g., Leiter et al. (1990) (*Proc. Natl. Acad. Sci. (USA)* **87**:3430-3434); vesicular stomatitis virus (see,

e.g., Agris et al. (1986) *Biochem.* **25**:6268-6275); herpes simplex (see, e.g., Gao et al. (1990) *Antimicrob. Agents Chem.* **34**:808-812); SV40 (see, e.g., Birg et al. (1990) (*Nucleic Acids Res.* **18**:2901-2908); and human papilloma virus (see, e.g., Storey et al. (1991) (*Nucleic Acids Res.* **19**:4109-4114). The use of synthetic oligonucleotides and their analogs as antiviral agents has recently been extensively reviewed by Agrawal (*Trends in Biotech.* (1992) **10**:152-158).

In addition, synthetic oligonucleotides have been used to inhibit a variety of non-viral pathogens, as well as to selectively inhibit the expression of certain cellular genes. Thus, the utility of synthetic oligonucleotides as agents to inhibit virus propagation, propagation of non-viral, pathogens and selective expression of cellular genes has been well established.

Improved oligonucleotides have more recently been developed that have greater efficacy in inhibiting such viruses, pathogens and selective gene expression. Some of these oligonucleotides having modifications in their internucleotide linkages have been shown to be more effective than their unmodified counterparts. For example, Agrawal et al. (*Proc. Natl. Acad. Sci. (USA)* (1988) **85**:7079-7083) teaches that oligonucleotide phosphorothioates and certain oligonucleotide phosphoramidates are more effective at inhibiting HIV-1 than conventional phosphodiester-linked oligodeoxynucleotides. Agrawal et al. (*Proc. Natl.*

Acad. Sci. (USA) (1989) **86**:7790-7794) discloses the advantage of oligonucleotide phosphorothioates in inhibiting HIV-1 in early and chronically infected cells.

In addition, chimeric oligonucleotides having more than one type of internucleotide linkage within the oligonucleotide have been developed. Pederson et al. (U.S. Patent Nos. 5,149,797 and 5,220,007 discloses chimeric oligonucleotides having an oligonucleotide phosphodiester or oligonucleotide phosphorothioate core sequence flanked by nucleotide methylphosphonates or phosphoramidates. Furdon et al. (*Nucleic Acids Res.* (1989) **17**:9193-9204) discloses chimeric oligonucleotides having regions of oligonucleotide phosphodiesters in addition to either oligonucleotide phosphorothioate or methylphosphonate regions. Quartin et al. (*Nucleic Acids Res.* (1989) **17**:7523-7562) discloses chimeric oligonucleotides having regions of oligonucleotide phosphodiesters and oligonucleotide methylphosphonates. Inoue et al. (*FEBS Lett.* (1987) **215**:237-250) discloses chimeric oligonucleotides having regions of deoxyribonucleotides and 2'-O-methyl-ribonucleotides.

Many of these modified oligonucleotides have contributed to improving the potential efficacy of the antisense oligonucleotide therapeutic approach. However, certain deficiencies remain in the known oligonucleotides, and these deficiencies can limit the effectiveness of such

oligonucleotides as therapeutic agents. For example, Wickstrom (*J. Biochem. Biophys. Meth.* (1986) **13**:97-102) teaches that oligonucleotide phosphodiesters are susceptible to nuclease-mediated degradation, thereby limiting their bioavailability *in vivo*. Agrawal et al. (*Proc. Natl. Acad. Sci. (USA)* (1990) **87**:1401-1405) teaches that oligonucleotide phosphoramidates or methylphosphonates when hybridized to RNA do not activate RNase H, the activation of which can be important to the function of antisense oligonucleotides. Thus, a need for methods of controlling gene expression exists which uses oligonucleotides with improved therapeutic characteristics.

Several reports have been published on the development of phosphorothioate-linked oligonucleotides as potential anti-AIDS therapeutic agents. Although extensive studies on chemical and molecular mechanisms of oligonucleotides have demonstrated the potential value of this novel therapeutic strategy, little is known about the pharmacokinetics and metabolism of these compounds *in vivo*.

Recently, several preliminary studies on this topic have been published. Agrawal et al. (*Proc. Natl. Acad. Sci. (USA)* (1991) **88**:7595-7599) describes the intravenously and intraperitoneally administration to mice of a 20-mer phosphorothioate linked-oligonucleotide. In this study, approximately 30% of the administered dose

was excreted in the urine over the first 24 hours with accumulation preferentially in the liver and kidney. Plasma half-lives ranged from about 1 hour ($t_{1/2\alpha}$) and 40 hours ($t_{1/2\beta}$), respectively. Similar results have been reported in subsequent studies (Iversen (1991) *Anti-Cancer Drug Design* 6:531-538; Iversen (1994) *Antisense Res. Devel.* 4:43-52; and Sands (1994) *Mol. Pharm.* 45:932-943). However, stability problems may exist when oligonucleotides are administered intravenously and intraperitoneally.

Thus, there remains a need to develop more effective therapeutic methods of down-regulating the expression of genes which can be easily manipulated to fit the animal and condition to be treated, and the gene to be targeted. Preferably, these methods should be simple, painless, and precise in effecting the target gene.

SUMMARY OF THE INVENTION

The present invention provides a method of down-regulating the expression of a gene in an animal which involves the administration of an oligonucleotide complementary to the gene via an oral route, thereby bypassing the complications which may be experienced during intravenous and other modes of in vivo administration.

It has been discovered that certain oligonucleotides (with other than phosphodiester bonds and having at least one 2'-substituted ribonucleotide) are relatively stable *in vivo*

following oral administration to an animal, and that these molecules are successfully absorbed from the gastrointestinal tract and distributed to various body tissues. This discovery has been exploited to develop the present invention, which is a method of down-regulating the expression of a gene in an animal.

This method is also a means of examining the function of various genes in an animal, including those essential to animal development. Presently, gene function can only be examined by the arduous task of making a "knock out" animal such as a mouse. This task is difficult, time-consuming and cannot be accomplished for genes essential to animal development since the "knock out" would produce a lethal phenotype. The present invention overcomes the shortcomings of this model.

In the method of the invention, a pharmaceutical formulation containing an oligonucleotide complementary to the targeted gene is orally administered in a pharmaceutically acceptable carrier to the animal harboring the gene. The oligonucleotide inhibits the expression of the gene, thereby down-regulating its expression.

For purposes of the invention, the term "animal" is meant to encompass humans as well as other mammals, as well as reptiles amphibians, and insects. The term "oral administration" refers to the provision of the formulation via the mouth through ingestion, or via some other part of

the gastrointestinal system including the esophagus.

As used herein, the term "oligonucleotide" is meant to include polymers of two or more nucleotides or nucleotide analogs connected together via 5' to 3' internucleotide linkages which may include any linkages that are known in the antisense art. Such molecules have a 3' terminus and a 5' terminus. The particular oligonucleotide being administered include at least one 2'-substituted ribonucleotide, and are connected via non-phosphodiester internucleotide linkages.

For purposes of the invention, the term "2'-substituted oligonucleotide" refers to an oligonucleotide having a sugar attached to a chemical group other that a hydroxyl group at its 2' position. The 2'-OH of the ribose molecule can be substituted with -O-lower alkyl containing 1-6 carbon atoms, aryl or substituted aryl or allyl having 2-6 carbon atoms, e.g., 2'-O-allyl, 2'-O-aryl, 2'-O-alkyl (such as a 2'-O-methyl), 2'-halo, or 2'-amino, but not with 2'-H, wherein allyl, aryl, or alkyl groups may be unsubstituted or substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl or amino groups.

The term "non-phosphodiester-linked oligonucleotide" as used herein is an oligonucleotide in which all of its nucleotides are covalently linked via a synthetic linkage,

i.e., a linkage other than a phosphodiester between the 5' end of one nucleotide and the 3' end of another nucleotide in which the 5' nucleotide phosphate has been replaced with any number of chemical groups. Preferable synthetic linkages include alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphoramidites, phosphate esters, carbamates, carbonates, phosphate triesters, acetamidate, and carboxymethyl esters. In one preferred embodiment of the invention, the all of the nucleotides of the oligonucleotide comprises are linked via phosphorothioate and/or phosphorodithioate linkages.

In some embodiments of the invention, the oligonucleotides administered are modified. As used herein, the term "modified oligonucleotide" encompasses oligonucleotides with modified nucleic acid(s), base(s), and/or sugar(s) other than those found in nature. For example, a 3', 5'-substituted oligonucleotide is an oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position).

A modified oligonucleotide may also be one with added substituents such as diamines, cholestryl, or other lipophilic groups, or a capped species. In addition, unoxidized or partially oxidized oligonucleotides having a

substitution in one nonbridging oxygen per nucleotide in the molecule are also considered to be modified oligonucleotides. Also considered as modified oligonucleotides are oligonucleotides having nuclease resistance-conferring bulky substituents at their 3′ and/or 5′ end(s) and/or various other structural modifications not found *in vivo* without human intervention are also considered herein as modified.

In one preferred embodiment of the invention, the oligonucleotide administered includes at least one 2′-substituted ribonucleotide at its 3′ terminus. In some embodiments, all but four or five nucleotides at its 5′ terminus are 2′-substituted ribonucleotides, and in some embodiments, these four or five unsubstituted 5′ nucleotides are deoxyribonucleotides. In other aspects, the oligonucleotide has at least one 2′-substituted ribonucleotide at both its 3′ and 5′ termini, and in yet other embodiments, the oligonucleotide is composed of 2′-substituted ribonucleotides in all positions with the exception of at least four or five contiguous deoxyribonucleotide nucleotides in any interior position. Another aspect of the invention includes the administration of an oligonucleotide composed of nucleotides that are all 2′-substituted ribonucleotides. Particular embodiments include oligonucleotides having a 2′-O-alkyl-ribonucleotide such as a 2′-O methyl.

In another embodiment of the invention, the oligonucleotide administered has at least one

deoxyribonucleotide, and in a preferred embodiment, the oligonucleotide has at least four or five contiguous deoxyribonucleotides capable of activating RNase H.

The oligonucleotide administered is complementary to a gene of a virus, pathogenic organism, or a cellular gene in some embodiments of the invention. In some embodiments, the oligonucleotide is complementary to a gene of a virus involved in AIDS, oral or genital herpes, papilloma warts, influenza, foot and mouth disease, yellow fever, chicken pox, shingles, adult T-cell leukemia, Burkitt's lymphoma, nasopharyngeal carcinoma, or hepatitis. In one particular embodiment, the oligonucleotide is complementary to an HIV gene and includes about 15 to 26 nucleotides linked by phosphorothioate internucleotide linkages, at least one of the nucleotides at the 3' terminus being a 2'-substituted ribonucleotide, and at least four contiguous deoxyribonucleotides.

In another embodiment, the oligonucleotide is complementary to a gene encoding a protein in associated with Alzheimer's disease.

In yet other embodiments, the oligonucleotide is complementary to a gene encoding a protein expressed in a parasite that causes a parasitic disease such as amebiasis, Chagas' disease, toxoplasmosis, pneumocytosis, giardiasis, cryptoporidiosis, trichomoniasis, malaria,

ascariasis, filariasis, trichinosis, or schistosomiasis infections.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

FIG. 1 is a graphic representation showing the time course of radiolabelled, modified PS-oligonucleotide 1 in liver, kidney and plasma following the oral administration of radiolabelled, oligonucleotide.

FIG. 2A is an HPLC chromatograph of radiolabelled oligonucleotide standard;

FIG. 2B is an HPLC chromatogram of oligonucleotides extracted from plasma samples taken 12 hours after the administration of radiolabelled oligonucleotide;

FIG. 3A is an HPLC chromatogram of radiolabelled oligonucleotide standard;

FIG. 3B is an HPLC chromatogram of oligonucleotides extracted from rat liver 6 hours after the administration of radiolabelled oligonucleotide;

FIG. 3C is an HPLC chromatogram of oligonucleotides extracted from rat liver 24 hours after the administration of radiolabelled oligonucleotide;

FIG. 4 is a graphic representation demonstrating the course of urinary excretion of radioactivity in rats following the oral administration of radiolabelled oligonucleotide;

FIG. 5A is an HPLC chromatogram of radiolabelled oligonucleotide standard;

FIG. 5B is an HPLC chromatogram of oligonucleotides extracted from rat urine 6 hours after the administration of radiolabelled oligonucleotide;

FIG. 5C is an HPLC chromatogram of oligonucleotides extracted from rat urine 12 hours after the administration of radiolabelled oligonucleotide;

FIG. 6 is a graphic representation showing the course of radioactivity in the gastrointestinal tract and feces in rats following the oral administration of radiolabelled oligonucleotide;

FIG. 7 is an HPLC chromatogram of oligonucleotides extracted from rat stomach 1 hour, 3 hours, and 6 hours after the administration of radiolabelled oligonucleotide; and

FIG. 8 is an HPLC chromatogram of oligonucleotides extracted from rat large intestine 3 hours, 6 hours, and 12 hours after the administration of radiolabelled oligonucleotide.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. The issued U.S. patent, allowed patent applications, and articles cited herein are hereby incorporated by reference.

This invention provides a method of down-regulating the expression of a gene in an animal by the oral administration of an oligonucleotide whose nucleotide sequence is complementary to the targeted gene.

It is known that an oligonucleotide, called an "antisense oligonucleotide," can bind to a target single-stranded nucleic acid molecule according to the Watson-Crick or the Hoogsteen rule of base pairing, and in doing so, disrupt the function of the target by one of several mechanisms: by preventing the binding of factors required for normal transcription, splicing, or translation; by triggering the enzymatic destruction of mRNA by RNase H if a contiguous region of deoxyribonucleotides exists in the oligonucleotide, and/or by destroying the target via reactive groups attached directly to the antisense oligonucleotide.

Thus, because of the properties described above, such oligonucleotides are useful therapeutically by their ability to control or down-regulate the expression of a particular gene

in an animal, according to the method of the present invention.

The oligonucleotides useful in the method of the invention are at least 6 nucleotides in length, but are preferably 6 to 50 nucleotides long, with 15 to 30mers being the most common. They are composed of deoxyribonucleotides, ribonucleotides, or a combination of both, with the 5' end of one nucleotide and the 3' end of another nucleotide being covalently linked by non-phosphodiester internucleotide linkages. Such linkages include alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. Oligonucleotides with these linkages can be prepared according to known methods such as phosphoramidate or H-phosphonate chemistry which can be carried out manually or by an automated synthesizer as described by Brown (*A Brief History of Oligonucleotide Synthesis. Protocols for Oligonucleotides and Analogs, Methods in Molecular Biology* (1994) **20**:1-8). (See also, e.g., Sonveaux "Protecting Groups in Oligonucleotides Synthesis" in Agrawal (1994) *Methods in Molecular Biology* **26**:1-72; Uhlmann et al. (1990) *Chem. Rev.* **90**:543-583).

The oligonucleotides of the composition may also be modified in a number of ways without compromising their ability to hybridize to the target nucleic acid. Such modifications include,

for example, those which are internal or at the end(s) of the oligonucleotide molecule and include additions to the molecule of the internucleoside phosphate linkages, such as cholesteryl or diamine compounds with varying numbers of carbon residues between the amino groups and terminal ribose, deoxyribose and phosphate modifications which cleave, or crosslink to the opposite chains or to associated enzymes or other proteins which bind to the viral genome. Examples of such modified oligonucleotides include oligonucleotides with a modified base and/or sugar such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position). Other modified oligonucleotides are capped with a nuclease resistance-conferring bulky substituent at their 3' and/or 5' end(s), or have a substitution in one nonbridging oxygen per nucleotide. Such modifications can be at some or all of the internucleoside linkages, as well as at either or both ends of the oligonucleotide and/or in the interior of the molecule. For the preparation of such modified oligonucleotides, see, e.g., Agrawal (1994) *Methods in Molecular Biology* **26**; Uhlmann et al. (1990) *Chem. Rev.* **90**:543-583).

Oligonucleotides which are self-stabilized are also considered to be modified oligonucleotides useful in the methods of the invention (Tang et al. (1993) *Nucleic Acids Res.*

20:2729-2735). These oligonucleotides comprise two regions: a target hybridizing region; and a self-complementary region having an oligonucleotide sequence complementary to a nucleic acid sequence that is within the self-stabilized oligonucleotide.

The preparation of these unmodified and modified oligonucleotides is well known in the art (reviewed in Agrawal et al. (1992) *Trends Biotechnol. 10:152-158) (see, e.g.*, Uhlmann et al. (1990) *Chem. Rev.* 90:543-584; and (1987) *Tetrahedron. Lett.* 28:(31):3539-3542); Agrawal (1994) *Methods in Molecular Biology* 20:63-80).

These oligonucleotides are provided with additional stability by having non-phosphodiester internucleotide linkages such as alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphoramidites, phosphate esters, carbamates, carbonates, phosphate triesters, acetamidate, and carboxymethyl esters. Particularly useful oligonucleotides are linked with phosphorothioate and/or phosphorodithioate internucleoside linkages.

The oligonucleotides administered to the animal may be hybrid oligonucleotides in that they contain both deoxyribonucleotides and at least one 2' substituted ribonucleotide. For purposes of the invention, the term "2'-substituted" means substitution of the 2'-OH of the ribose molecule with, e.g., 2'-O-allyl, 2'-O-alkyl, 2'-halo, or

2'-amino, but not with 2'-H, wherein allyl, aryl, or alkyl groups may be unsubstituted or substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl or amino groups.

The hybrid DNA/RNA oligonucleotides useful in the method of the invention resist nucleolytic degradation, form stable duplexes with RNA or DNA, and preferably activate RNase H when hybridized with RNA. They may additionally include at least one unsubstituted ribonucleotide. For example, an oligonucleotide useful in the method of the invention may contain all deoxyribonucleotides with the exception of one 2' substituted ribonucleotide at the 3' terminus of the oligonucleotide.

Alternatively, the oligonucleotide may have at least one substituted ribonucleotide at both its 3' and 5' termini.

One preferred class of oligonucleotides useful in the method of the invention contains four or more deoxyribonucleotides in a contiguous block, so as to provide an activating segment for RNase H. In certain cases, more than one such activating segment will be present at any location within the oligonucleotide. There may be a majority of deoxyribonucleotides in oligonucleotides according to the invention. In fact, such oligonucleotides may have as many as all but one nucleotide being deoxyribonucleotides. Thus, a preferred oligonucleotide having from

about 2 to about 50 nucleotides or most preferably from about 12 to about 25 nucleotides, the number of deoxyribonucleotides present ranges from 1 to about 24. Other useful oligonucleotides may consist only of 2'-substituted ribonucleotides.

TABLE 1 lists some representative species of oligonucleotides which are useful in the method of the invention. 2'-substituted nucleotides are underscored.

TABLE 1

NO.	OLIGONUCLEOTIDE
1	CTCTCGCACCCATCTCTCTCCTTCU
2	CTCTCGCACCCATCTCTCTCCTUCU
3	CTCTCGCACCCATCTCTCTCCUUCU
4	CTCTCGCACCCATCTCUCUCCUUCU
5	CTCTCGCACCCAUCUCUCUCCUUCU
6	CTCTCGCACCCAUCUCUCUCCUUCU
7	CTCTCGCACCCAUCUCUCUCCUUCU
8	CUCUCGCACCCAUCUCUCUCCUUCU
9	CTCTCGCACCCATCTCTCTCCTTCU
10	CUCTCGCACCCATCTCTCTCCTTCU
11	CUCUCGCACCCATCTCTCTCCUUCU
12	CUCUCGCACCCATCTCUCUCCUUCU
13	CUCUCGCACCCAUCUCUCUCCUUCU
14	CUCUCGCACCCATCTCTCUCCUUCU
15	CTCTCGCACCCAUCUCUCUCCUUCU
16	CUCUCGCACCCAUCTCTCTCCUUCU
17	CUCUCGCACCCATCTCTCTCCUUCU
18	CUCTCGCACCCAUCUCUCUCCUUCU
19	CUCTCGCACCCATCTCTCUCCUUCU

The 2' substituted ribonucleotide(s) in the oligonucleotide may contain at the 2' position of the ribose, a -O-lower alkyl containing 1-6 carbon atoms, aryl or substituted aryl or allyl having 2-6 carbon atoms e.g., 2'-O-allyl, 2'-O-aryl, 2'-O-alkyl, 2'-halo, or 2'-amino, but not with 2'-H, wherein allyl, aryl, or alkyl groups may be unsubstituted or substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl or amino groups. Useful substituted ribonucleotides are 2'-O-alkyls such as 2'-O-methyl.

Preferably, oligonucleotides according to the invention will range from about 2 to about 50 nucleotides in length, and most preferably from about 15 to about 25 nucleotides in length. Thus, in this preferred case, oligonucleotides according to the invention will have from 14 to 24 non-phosphodiester internucleotide linkages.

The oligonucleotides according to the invention are effective in inhibiting the expression of various genes in viruses, pathogenic organisms, or in inhibiting the expression of cellular genes. The ability to inhibit such agents is clearly important to the treatment of a variety of disease states. Thus, oligonucleotides according to the method of the invention have a nucleotide sequence which is complementary to a nucleic acid sequence that is from a virus, a pathogenic organism or a cellular gene. Preferably such oligonucleotides are from about 6 to about 50 nucleotides in length.

For purposes of the invention, the term "oligonucleotide sequence that is complementary to a nucleic acid sequence" is intended to mean an oligonucleotide sequence that binds to the target nucleic acid sequence under physiological conditions, e.g., by Watson-Crick base pairing (interaction between oligonucleotide and single-stranded nucleic acid) or by Hoogsteen base pairing (interaction between oligonucleotide and double-stranded nucleic acid) or by any other means including in the case of a oligonucleotide binding to RNA, pseudoknot formation. Such binding (by Watson Crick base pairing) under physiological conditions is measured as a practical matter by observing interference with the function of the nucleic acid sequence.

The nucleic acid sequence to which an oligonucleotide according to the invention is complementary will vary, depending upon the gene to be down-regulated. In some cases, the target gene or nucleic acid sequence will be a virus nucleic acid sequence. The use of antisense oligonucleotides to inhibit various viruses is well known (reviewed in Agrawal (1992) *Trends in Biotech.* **10**:152-158). Viral nucleic acid sequences that are complementary to effective antisense oligonucleotides have been described for many viruses, including human immunodeficiency virus type 1 (HIV-1) (U.S. Patent No. 4,806,463), herpes simplex virus (U.S. Patent No. 4,689,320), influenza virus (U.S. Patent No. 5,194,428), and human papilloma virus (Storey et al. (1991) *Nucleic Acids Res.* **19**:4109-4114). Sequences complementary

to any of these nucleic acid sequences can be used for oligonucleotides according to the invention, as can be oligonucleotide sequences complementary to nucleic acid sequences from any other virus. Additional viruses that have known nucleic acid sequences against which antisense oligonucleotides can be prepared include, but are not limited to, foot and mouth disease virus (see, Robertson et al. (1985) *J. Virol.* **54**:651; Harris et al. *(1980) Virol.* **36**:659), yellow fever virus (see Rice et al. (1985) *Science* **229**:726), varicella-zoster virus (see, Davison and Scott (1986) *J. Gen. Virol.* **67**:2279), Epstein-Barr virus, cytomegalovirus, respiratory syncytial virus (RSV), and cucumber mosaic virus (see Richards et al. (1978) *Virol.* **89**:395).

For example, an oligonucleotide has been designed which is complementary to a portion of the HIV-1 gene, and as such, has significant anti-HIV effects (Agrawal (1992) *Antisense Res. Development* **2**:261-266). The target of this oligonucleotide has been found to be conserved among various HIV-1 isolates. It is 56% G + C rich, water soluble, and relatively stable under physiological conditions. This oligonucleotide binds to a complementary RNA target under physiological conditions, with the T of the duplex approximately being 56°C. The antiviral activity of this oligonucleotide has been tested in several models, including acutely and chronically infected CEM cells, long-term cultures mimicking *in vivo* conditions, human peripheral blood lymphocytes and

macrophages, and isolates from HIV-1 infected patients (Lisziewicz et al. (*Proc. Natl. Acad. Sci. (USA)* (1992) **89**:11209-11213); Lisziewicz et al. (*Proc. Natl. Acad. Sci. (USA)* (1993) **90**:3860-3864); Lisziewicz et al. (*Proc. Natl. Acad. Sci. (USA)* (1994) **91**:7942-7946); Agrawal et al. (*J. Ther. Biotech*) in press).

The oligonucleotides according to the invention alternatively can have an oligonucleotide sequence complementary to a nucleic acid sequence of a pathogenic organism. The nucleic acid sequences of many pathogenic organisms have been described, including the malaria organism, *Plasmodium falciparum*, and many pathogenic bacteria. Oligonucleotide sequences complementary to nucleic acid sequences from any such pathogenic organism can be used in oligonucleotides according to the invention. Examples of pathogenic eucaryotes having known nucleic acid sequences against which antisense oligonucleotides can be prepared include *Trypanosom abrucei gambiense* and *Leishmania* (See Campbell et al., *Nature* **311**:350 (1984)), *Fasciola hepatica* (See Zurita et al., *Proc. Natl. Acad. Sci. USA* **84**:2340 (1987).

Antifungal oligonucleotides can be prepared using a target hybridizing region having an oligonucleotide sequence that is complementary to a nucleic acid sequence from, e.g., the chitin synthetase gene, and antibacterial oligonucleotides can be prepared using, e.g., the alanine racemase gene. Among fungal diseases that may be treatable by the method of treatment

according to the invention are candidiasis, histoplasmosis, cryptococcocis, blastomycosis, aspergillosis, sporotrichosis, chromomycosis, dermatophytosis, and coccidioidomycosis. The method might also be used to treat rickettsial diseases (e.g., typhus, Rocky Mountain spotted fever), as well as sexually transmitted diseases caused by Chlamydia trachomatis or Lymphogranuloma venereum. A variety of parasitic diseases may be treated by the method according to the invention, including amebiasis, Chagas' disease, toxoplasmosis, pneumocystosis, giardiasis, cryptosporidiosis, trichomoniasis, and Pneumocystis carini pneumonia; also worm (helminthic) diseases such as ascariasis, filariasis, trichinosis, schistosomiasis and nematode or cestode infections. Malaria may be treated by the method of treatment of the invention regardless of whether it is caused by P. falcip arum, P. vivas, P. orale, or P. malariae.

The infectious diseases identified above may all be treated by the method of treatment according to the invention because the infectious agents for these diseases are known and thus oligonucleotides according to the invention can be prepared, having oligonucleotide sequence that is complementary to a nucleic acid sequence that is an essential nucleic acid sequence for the propagation of the infectious agent, such as an essential gene.

Other disease states or conditions that may be treatable by the method according to the

invention are those which result from an abnormal expression or product of a cellular gene. These conditions may be treated by administration of oligonucleotides according to the invention, and have been discussed earlier in this disclosure.

Other oligonucleotides according to the invention can have a nucleotide sequence complementary to a cellular gene or gene transcript, the abnormal expression or product of which results in a disease state. The nucleic acid sequences of several such cellular genes have been described, including prion protein (Stahl et al. (1991) *FASEB J.* 5:2799-2807), the amyloid-like protein associated with Alzheimer's disease (U.S. Patent No. 5,015,570), and various well-known oncogenes and proto-oncogenes, such as c-*myb*, c-*myc*, c-*abl*, and n-*ras*. In addition, oligonucleotides that inhibit the synthesis of structural proteins or enzymes involved largely or exclusively in spermatogenesis, sperm motility, the binding of the sperm to the egg or any other step affecting sperm viability may be used as contraceptives. Similarly, contraceptives for women may be oligonucleotides that inhibit proteins or enzymes involved in ovulation, fertilization, implantation or in the biosynthesis of hormones involved in those processes.

Hypertension may be controlled by oligonucleotides that down-regulate the synthesis of angiotensin converting enzyme or related enzymes in the renin/angiotensin system. Platelet aggregation may be controlled by suppression of

the synthesis of enzymes necessary for the synthesis of thromboxane A2 for use in myocardial and cerebral circulatory disorders, infarcts, arteriosclerosis, embolism and thrombosis. Deposition of cholesterol in arterial wall may be inhibited by suppression of the synthesis of fatty acid co-enzyme A: cholesterol acyl transferase in arteriosclerosis. Inhibition of the synthesis of cholinephosphotransferase may be useful in hypolipidemia.

There are numerous neural disorders in which hybridization arrest may be used to reduce or eliminate adverse effects of the disorder. For example, suppression of the synthesis of monoamine oxidase may be used in Parkinson's disease. Suppression of catechol o-methyl transferase may be used to treat depression; and suppression of indole N-methyl transferase may be used in treating schizophrenia.

Suppression of selected enzymes in the arachidonic acid cascade which leads to prostaglandins and leukotrienes may be useful in the control of platelet aggregation, allergy, inflammation, pain and asthma.

Suppression of the protein expressed by the multidrug resistance (*mdr*-1) gene, which can be responsible for development of resistance of tumors to a variety of anti-cancer drugs and is a major impediment in chemotherapy may prove to be beneficial in the treatment of cancer. Oligonucleotide sequences complementary to nucleic

acid sequences from any of these genes can be used for oligonucleotides according to the invention, as can be oligonucleotide sequences complementary to any other cellular gene transcript, the abnormal expression or product of which results in a disease state.

The oligonucleotides described herein are administered orally or enterally to the animal subject in the form of therapeutic pharmaceutical formulations that are effective for treating virus infection, infections by pathogenic organisms, or disease resulting from abnormal gene expression or from the expression of an abnormal gene product. In some aspects or the method according to the invention, the oligonucleotides are administered in conjunction with other therapeutic agents, e.g., AZT in the case of AIDS.

The therapeutic pharmaceutical formulation containing the oligonucleotide includes a physiologically acceptable carrier, such as an inert diluent or an assimilable edible carrier with which the peptide is administered. Suitable formulations that include pharmaceutically acceptable excipients for introducing compounds to the bloodstream by other than injection routes can be found in *Remington's Pharmaceutical Sciences* (18th ed.) (Genarro, ed. (1990) Mack Publishing Co., Easton, PA). The oligonucleotide and other ingredients may be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the individual's diet. The oligonucleotide may be incorporated with

excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. When the oligonucleotide is administered orally, it may be mixed with other food forms and pharmaceutically acceptable flavor enhancers. When the oligonucleotide is administered enterally, they may be introduced in a solid, semi-solid, suspension, or emulsion form and may be compounded with any number of well-known, pharmaceutically acceptable additives. Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are also contemplated such as those described in U.S. Patent Nos. 4,704,295, 4,556,552, 4,309,404, and 4,309,406.

The amount of oligonucleotide in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit contains between from about 50 micrograms to about 200 mg per kg body weight of the animal, with 10 mg to 100 mg per kg being most preferable.

It will be appreciated that the unit content of active ingredient or ingredients contained in an individual dose of each dosage form need not in itself constitute an effective amount since the necessary effective amount can be reached by administration of a plurality of dosage units

(such as capsules or tablets or combinations thereof).

In order to determine if the oligonucleotide administered according to the method of the invention is absorbed into body tissues, and if so, in which tissues absorption occurs, the following study was performed. Samples of various body tissues were analyzed for radioactivity at increasing hours after oral administration of a radioactively labelled oligonucleotide. FIG. 1 illustrates the plasma, liver, and kidney concentration-time course of an oligonucleotide equivalents after oral administration of the radiolabelled oligonucleotide. These results demonstrate that the drug is absorbed through gastrointestinal tract and accumulated in the kidney and the liver.

The chemical form of radioactivity in plasma was further evaluated by HPLC, demonstrating the presence of both intact oligonucleotide (A) as well as metabolites (B) 12 hours after oral administration (FIG. 2B). Intact oligonucleotide was also detected in liver 6 hour (FIG. 3B) and 12 hours (FIG. 3C) after administration. Radioactivity in brain, thymus, heart, lung, liver, kidney, adrenals, stomach, small intestine, large intestine, skeletal muscle, testes, thyroid, epidermis, whole eye, and bone marrow was detectable 48 hours after oral administration of the radiolabelled oligonucleotide.

Further evidence to support the absorption of the oligonucleotide comes from urine sample analysis after radioactively labelled oligonucleotide was orally administered. FIG. 4 shows the cumulative excretion of the radioactively labelled oligonucleotide into the urine over 48 hr following the administration of radiolabelled oligonucleotide. That the oligonucleotide continues to be excreted in the urine over time implies that other tissues had absorbed it, and that the body was capable of absorption for an extended period of time. FIGS. 5B and 5C demonstrate that although the majority of radioactivity in urine was present as degradative products, intact oligonucleotide was also detected, demonstrating that this oligonucleotide is absorbed intact.

To determine the level of bioavailability of oligonucleotides following oral administration the level of the oligonucleotide in the gastrointestinal tract (stomach and intestine) and feces was measured. FIG. 6 shows that approximately 80% of administered oligonucleotide remained or was excreted in feces, indicating that 20% of administered oligonucleotide was absorbed. This oligonucleotide was stable in stomach; no significant degradative products in stomach contents were detected six hours after oral administration (FIG. 7). The majority of administered oligonucleotide in the contents of the large intestine were also present as the intact compound (FIG. 8).

Thus, using the method of the invention, successful absorption of oligonucleotides was accomplished through the gastrointestinal tract and distributed throughout the body. Intact oligonucleotides were detected in plasma and various tissues and excreted into the urine. These results demonstrate that oral administration is a potential means for delivery of oligonucleotides as therapeutic agents.

The following examples illustrate the preferred modes of making and practicing the present invention, but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar results.

EXAMPLES

1. Synthesis and Analysis of Oligonucleotide

A hybrid 25-mer phosphorothioate-linked oligonucleotide having SEQ ID NO:1 and containing 2'-0-methyl ribonucleotide 3' and 5' sequences and a deoxyribonucleotide interior was synthesized, purified, and analyzed as follows.

Unmodified phosphorothioate deoxynucleosides were synthesized on CPG on a 5-6 μmole scale on an automated synthesizer (model 8700, Millipore, Bedford, MA) using the H-phosphonate approach described in U.S. Patent No. 5,149,798. Deoxynucleoside H-phosphonates were obtained from Millipore (Bedford, MA).

2'-O-methyl ribonucleotide H-phosphonates or phosphorothioates were synthesized by standard procedures (see, e.g., "Protocols for Oligonucleotides and Analogs" in *Meth. Mol. Biol.* (1993) volume 20) or commercially obtained (e.g., from Glenn Research, Sterling, VA and Clontech, Palo Alto, CA). Segments of oligonucleotides containing 2'-O-methyl nucleoside(s) were assembled by using 2'-O-methyl ribonucleoside H-phosphonates or phosphorothioates for the desired cycles. Similarly, segments of oligonucleotides containing deoxyribonucleosides were assembled by using deoxynucleoside H-phosphonates for the desired cycles. After assembly, CPG bound oligonucleotide H-phosphonate was oxidized with sulfur to generate the phosphorothioate linkage. Oligonucleotides were then deprotected in concentrated NH_4OH at 40°C for 48 hours.

Crude oligonucleotide (about 500 A_{260} units) was analyzed on reverse low pressure chromatography on a C_{18} reversed phase medium. The DMT group was removed by treatment with 80% aqueous acetic acid, then the oligonucleotides were dialyzed against distilled water and lyophilized.

2. Radioactive Labelling of Oligonucleotide

To obtain ^{35}S-labelled oligonucleotide, synthesis was carried out in two steps. The first 19 nucleotides of the sequence SEQ ID NO:1) from its 3'-end were assembled using the β-cyanoethyl-phosphoramidite approach (see, Beaucage in *Protocols*

for Oligonucleotides and Analogs (Agrawal, ed.), Humana Press, (1993), pp. 33-61). The last six nucleotides were assembled using the H-phosphonate approach (see, Froehler in *Protocols for Oligonucleotides and Analogs* (Agrawal, ed.) Humana Press, 1993, pp. 63-80). Controlled pore glass (CPG) support-bound oligonucleotide (30 mg of CPG; approximately 1 μM) containing five H-phosphonate linkage was oxidized with $^{35}S_8$ (4 mCi, 1 Ci/mg, Amersham; 1 Ci = 37 GBq) in 60 ml carbon disulfide/pyridine/triethylamine (10:10:1). The oxidation reaction was performed at room temperature for 1 hr with occasional shaking. Then 2 μl, 5 μl, and 200 μl of 5% cold sulfur ($^{32}S_8$) in same solvent mixture was added every 30 min to complete the oxidation. The solution was removed and the CPG support was washed with carbon disulfide/pyridine/ triethylamine (10:10:1) (3 x 500 μl) and with acetonitrile (3 x 700 μl). The product was deprotected in concentrated ammonium hydroxide (55°C, 14 hr) and evaporated. The resultant product was purified by polyacrylamide gel electrophoresis (20% polyacrylamide containing 7 M urea). The desired band was excised under UV shadowing and the PS-oligonucleotide was extracted from the gel and desalted with a Sep-Pak C18 cartridge (Waters) and Sephadex G-15 column. The yield was 20 A_{260} units (600 μg; specific activity, 1 μCi/μg).

3. Animals and Treatment

Male Sprague-Dawley rats (100-120 g, Harlan Laboratories, Indianapolis, IN) were used in the

study. The animals were fed with commercial diet and water *ad libitum* for 1 week prior to the study.

Animals were dosed via gavage at a dose of 50 mg/kg. Unlabelled and ^{35}S-labelled oligonucleotides were dissolved in physiological saline (0.9% NaCl) in a concentration of 25 mg/ml. Doses were based on the pretreatment body weight and rounded to the nearest 0.01 ml. After dosing, each animal was placed in a metabolism cage and fed with commercial diet and water *ad libitum*. Total voided urine was collected and each metabolism cage was then washed following the collection intervals. Total excreted feces was collected from each animal at various timepoints and feces samples were homogenized prior to quantitation of radioactivity. Blood samples were collected in heparinized tubes from animals at the various timepoints. Plasma was separated by centrifugation. Animals were euthanized by exsanguination under sodium pentobarbital anesthesia. Following euthanasia, the tissues were collected from each animal. All tissues/organs were trimmed of extraneous fat or connective tissue, emptied and cleaned of all contents, individually weighed, and the weights recorded.

4. Total Radioactivity Measurements

The total radioactivities in tissues and body fluids were determined by liquid scintillation spectrometry (LS 6000TA, Beckman, Irvine, CA). In brief, biological fluids (plasma, 50-100 µl;

urine, 50-100 μl) were mixed with 6 ml scintillation solvent (Budget-Solve, RPI, Mt. Prospect, IL) to determine total radioactivity. Feces were ground and weighed prior to being homogenized in a 9-fold volume of 0.9% NaCl saline. An aliquot of the homogenate (100 μl) was mixed with solubilizer (TS-2, RPI, Mt. Prospect, IL) and then with scintillation solvent (6 ml) to permit quantitation of total radioactivity.

Following their removal, tissues were immediately blotted on Whatman No. 1 filter paper and weighed prior to being homogenized in 0.9% NaCl saline (3-5 ml per gram of wet weight). The resulting homogenate (100 μl) was mixed with solubilizer (TS-2, RPI, Mt. Prospect, IL) and then with scintillation solvent (6 ml) to determine total radioactivity. The volume of 0.9% NaCl saline added to each tissue sample was recorded. The homogenized tissues/organs were kept frozen at ≤-70°C until the use for further analysis.

5. HPLC Analysis

The radioactivity in urine was analyzed by paired-ion HPLC using a modification of the method described essentially by Sands et al. (*Mol. Pharm.* (1994) **45**:932-943). Urine samples were centrifuged and passed through a 0.2-μm Acro filter (Gelman, Ann Arbor, MI) prior to analysis. Hybrid oligonucleotide and metabolites in plasma samples were extracted using the above methods in sample preparation for PAGE. A Microsorb MV-C4 column (Rainin Instruments, Woburn, MA) was

employed in HPLC using a Hewlett Packard 1050 HPLC with a quaternary pump for gradient making. Mobile phase included two buffers; Buffer A was 5 mM-A reagent (Waters Co., Bedford, MA) in water and Buffer B was 4:1 (v/v) Acetonitrile (Fisher)/water. The column was eluted at a flow rate of 1.5 ml/min, using the following gradient: (1) 0-4 min, 0% buffer B; (2) 4-15 min 0-35% Buffer B; and (3) 15-70 min 35%-80% Buffer B. The column was equilibrated with Buffer A for at least 30 min prior to the next run. By using a RediFrac fraction collector (Pharmacia LKB Biotechnology, Piscataway, NJ), 1-min fractions (1.5 ml) were collected into 7-ml scintillation vials and mixed with 5 ml scintillation solvent to determine radioactivity in each fraction.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Hybridon, Inc.

(ii) TITLE OF INVENTION: A Method Of Down-Regulating Gene Expression

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Lappin & Kusmer
(B) STREET: 200 State Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: USA
(F) ZIP: 02109

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kerner, Ann-Louise
(B) REGISTRATION NUMBER: 33,523
(C) REFERENCE/DOCKET NUMBER: HYZ-030PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617-330-1300
(B) TELEFAX: 617-330-1311

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA/RNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

5 25

CUCUCGCACC CATCTCTCTC CUUCU

What is claimed is:

1. A method of down-regulating the expression of a gene in an animal,

the method comprising the step of orally administering a pharmaceutical formulation comprising an oligonucleotide complementary to the gene in a pharmaceutically acceptable carrier,

the oligonucleotide having non-phosphodiester internucleotide linkages and comprising at least one 2'-substituted ribonucleotide,

the oligonucleotide inhibiting the expression of a product of the gene, thereby down-regulating the expression of the gene.

2. The method of claim 1 wherein the oligonucleotide has 3' and 5' termini and the 2'-substituted ribonucleotide is at the 3' terminus.

3. The method of claim 2 wherein the oligonucleotide further comprises at least one 2'-substituted ribonucleotide at the 5' terminus.

4. The method of claim 1 wherein all of the nucleotides in the oligonucleotide are 2'-substituted ribonucleotides.

5. The method of claim 1 wherein the 2'-substituted ribonucleotide is a 2'-O-alkyl-ribonucleotide.

6. The method of claim 1 wherein the oligonucleotide comprises at least one deoxyribonucleotide.

7. The method of claim 6 wherein the oligonucleotide comprises a region of at least four contiguous deoxyribonucleotides capable of activating RNase H activity.

8. The method of claim 1 wherein the oligonucleotide comprises an internucleotide linkage selected from the group consisting of alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphoramidites, phosphate esters, carbamates, carbonates, phosphate triesters, acetamidate, and carboxymethyl esters.

9. The method of claim 8 wherein essentially all of the nucleotides are linked via phosphorothioate or phosphorodithioate internucleotide linkages.

10. The method of claim 1 wherein the oligonucleotide is modified.

11. The method of claim 1 wherein the oligonucleotide is complementary to a gene of a virus, pathogenic organism, or a cellular gene.

12. The method of claim 11 wherein the oligonucleotide is complementary to a gene of a virus involved in a disease selected from the group consisting of AIDS, oral and genital herpes, papilloma warts, influenza, foot and mouth disease, yellow fever, chicken pox, shingles, adult T-cell leukemia, Burkitt's lymphoma, nasopharyngeal carcinoma, and hepatitis.

13. The method of claim 1 wherein the oligonucleotide is complementary to a gene encoding a protein associated with Alzheimer's disease.

14. The method of claim 1 wherein the oligonucleotide is complementary to a gene encoding a protein in a parasite causing a parasitic disease selected from the group consisting of amebiasis, Chagas' disease, toxoplasmosis, pneumocytosis, giardiasis, cryptoporidiosis, trichomoniasis, malaria, ascariasis, filariasis, trichinosis, schistosomiasis infections.

15. The method of claim 1 wherein the oligonucleotide is complementary to an HIV gene and comprises about 15 to 26 nucleotides linked by phosphorothioate internucleotide linkages, at least one of the nucleotides at the 3' terminus being a 2'-substituted ribonucleotide, and at least four nucleotides being contiguous deoxyribonucleotides.

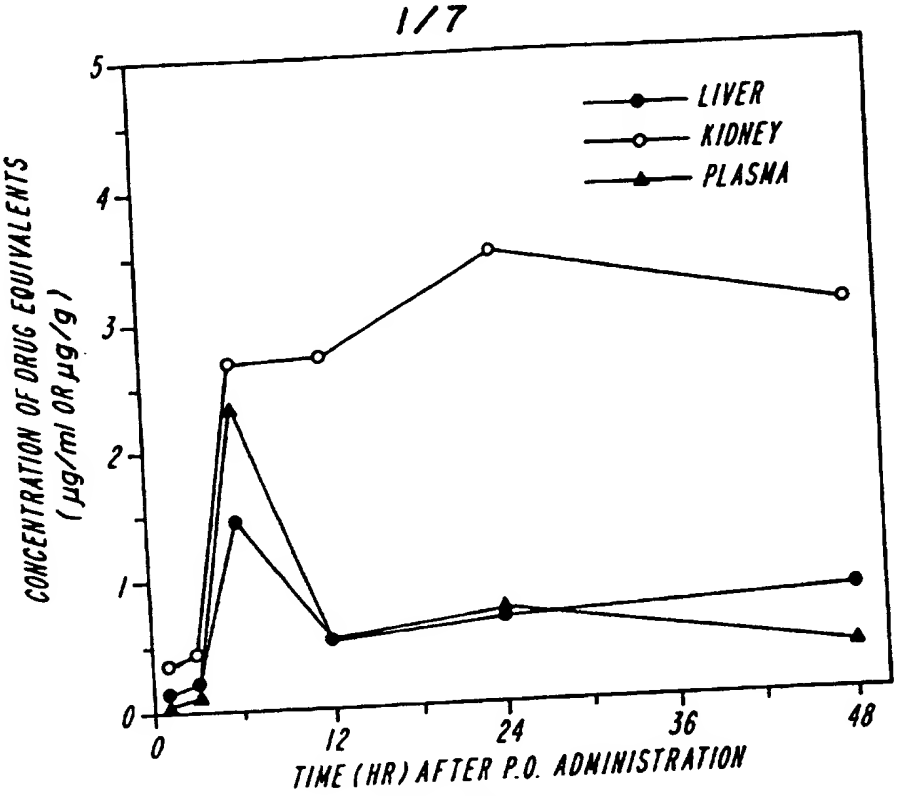
LIVER
KIDNEY
PLASMA
CONCENTRATION OF DRUG EQUIVALENTS
(μg/ml OR μg/g)
0
1
2
3
4
5
0
12
24
36
48
TIME (HR) AFTER P.O. ADMINISTRATION

FIG. 1

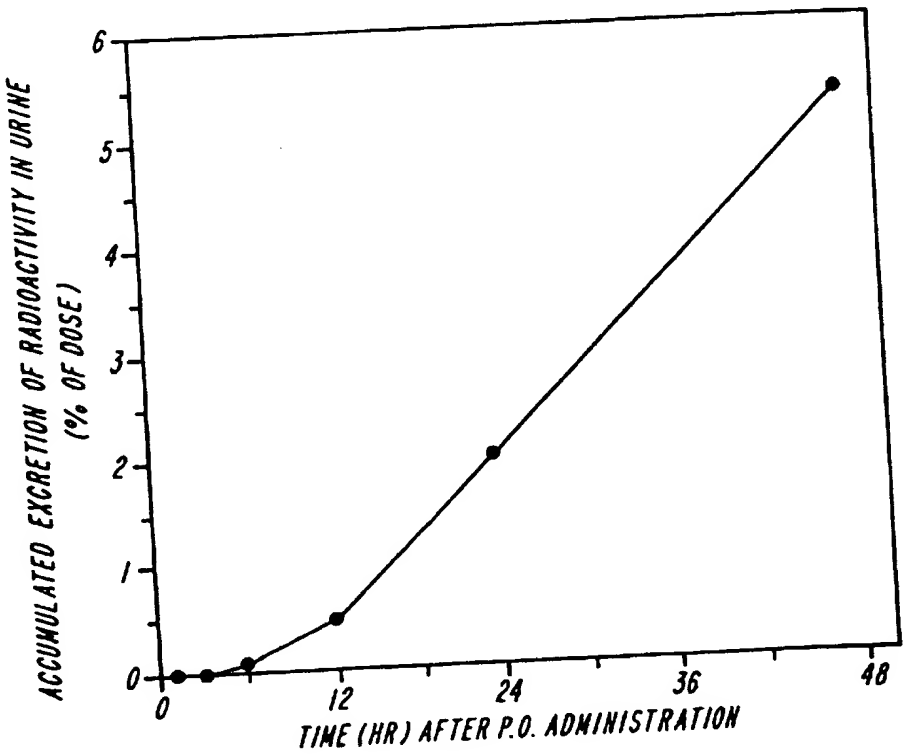
ACCUMULATED EXCRETION OF RADIOACTIVITY IN URINE
(% OF DOSE)
0
1
2
3
4
5
6
0
12
24
36
48
TIME (HR) AFTER P.O. ADMINISTRATION

FIG. 4

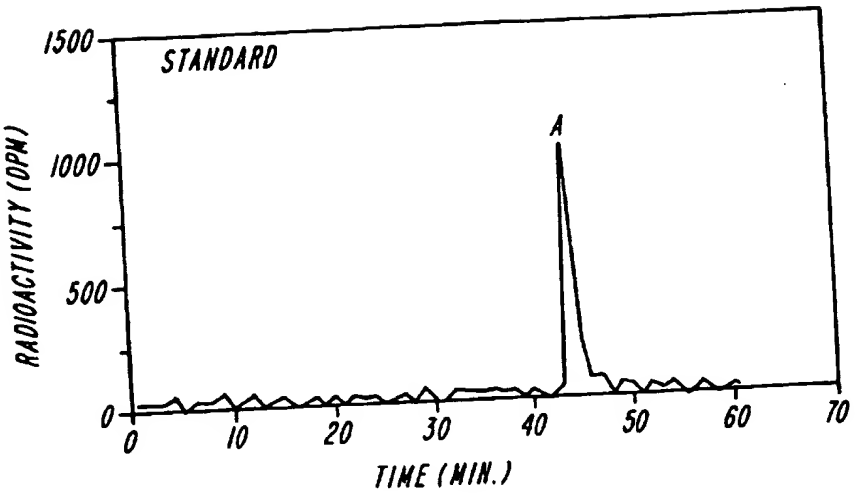
STANDARD
A
RADIOACTIVITY (DPM)
1500
1000
500
0
0
10
20
30
40
50
60
70
TIME (MIN.)

FIG. 2A

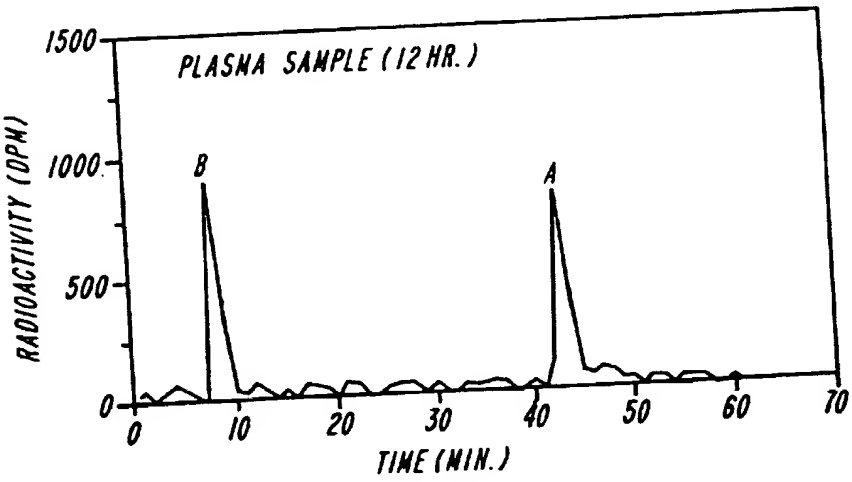
PLASMA SAMPLE (12 HR.)
B
A
RADIOACTIVITY (DPM)
1500
1000
500
0
0
10
20
30
40
50
60
70
TIME (MIN.)

FIG. 2B

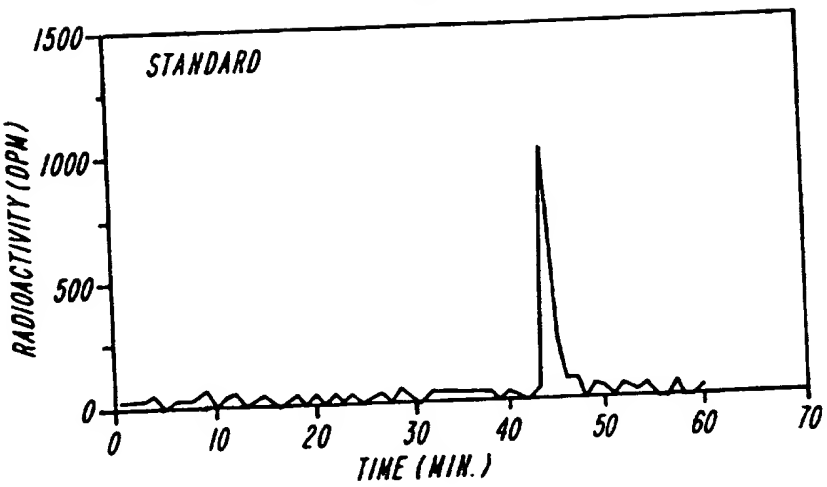

FIG. 3A

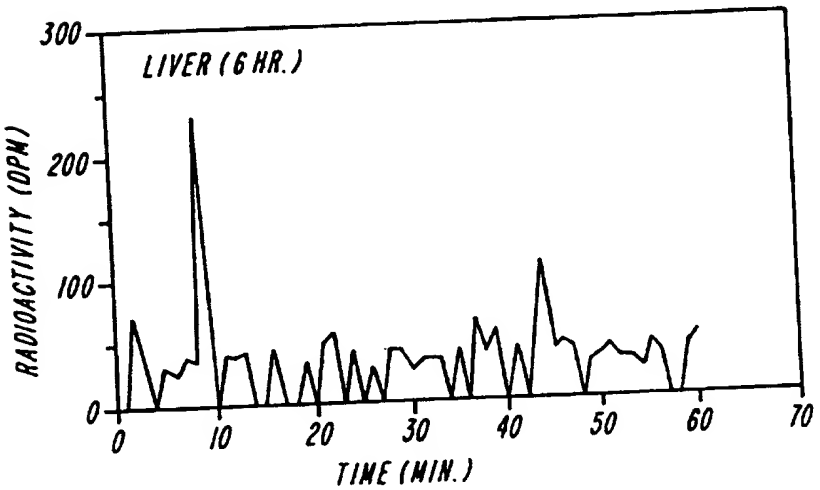

FIG. 3B

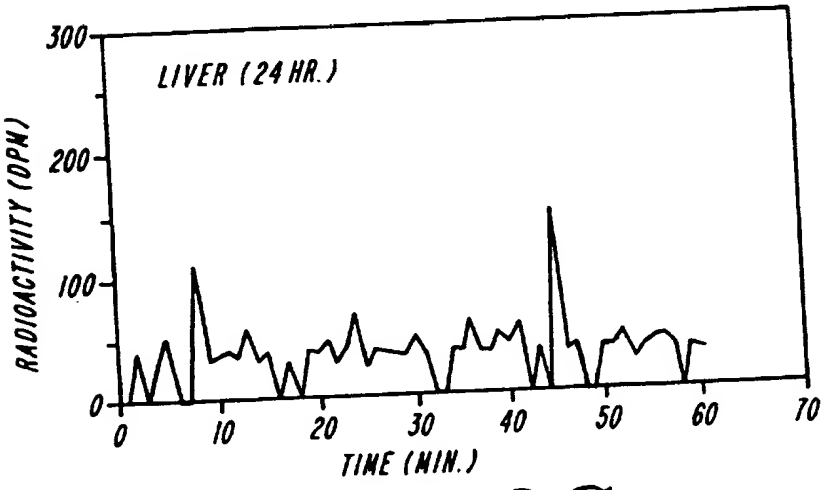

FIG. 3C

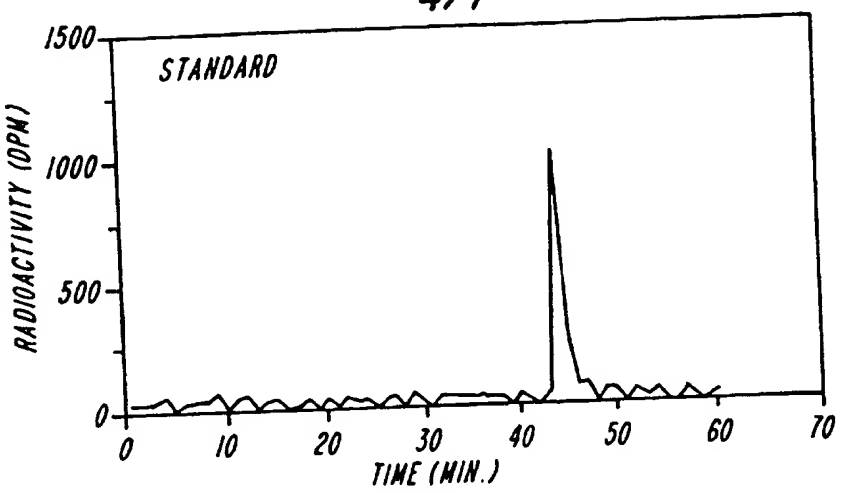

FIG. 5A

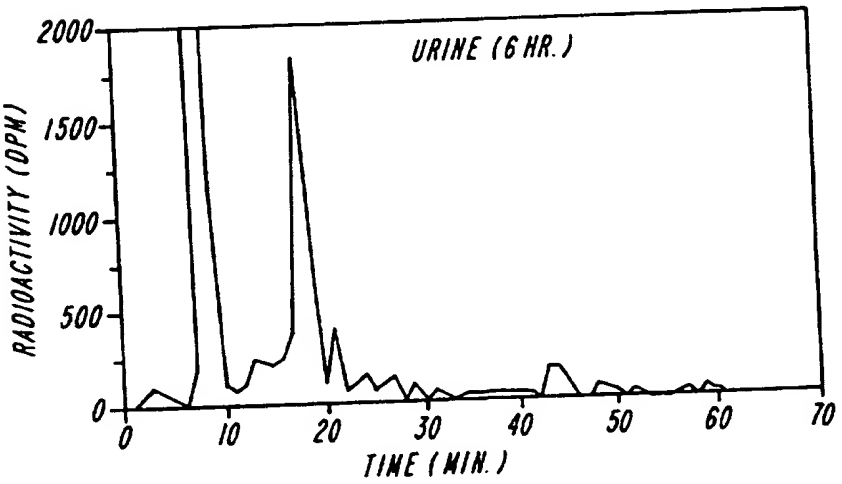

FIG. 5B

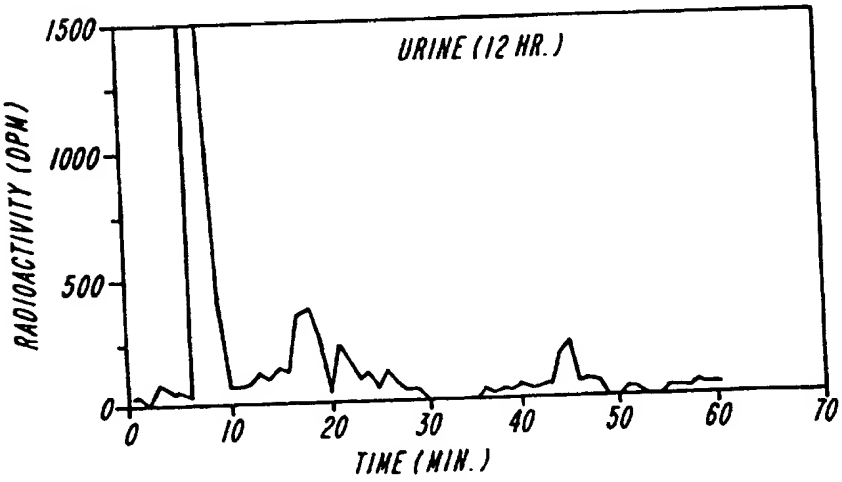

FIG. 5C

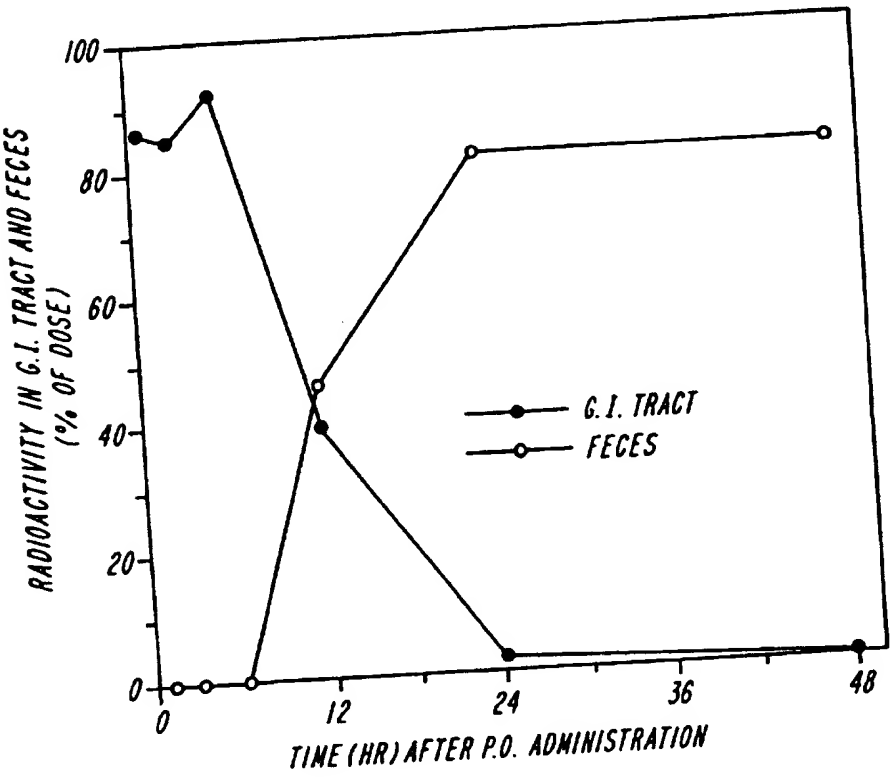
100
80
60
40
20
0
0
12
24
36
48
RADIOACTIVITY IN G.I. TRACT AND FECES (% OF DOSE)
TIME (HR) AFTER P.O. ADMINISTRATION
G.I. TRACT
FECES

FIG. 6

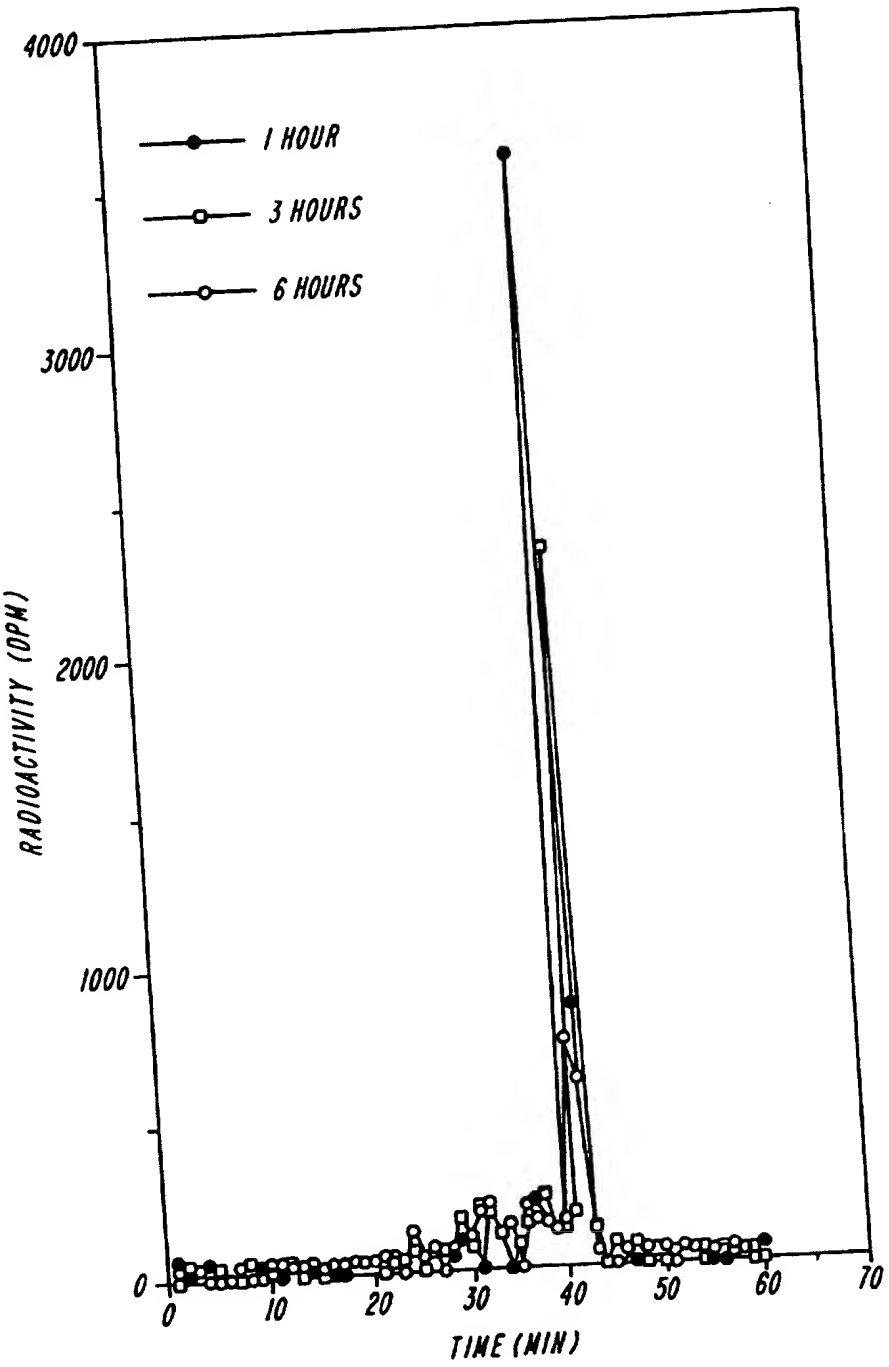
4000
3000
2000
1000
0
1 HOUR
3 HOURS
6 HOURS
RADIOACTIVITY (DPM)
0
10
20
30
40
50
60
70
TIME (MIN)

FIG. 7

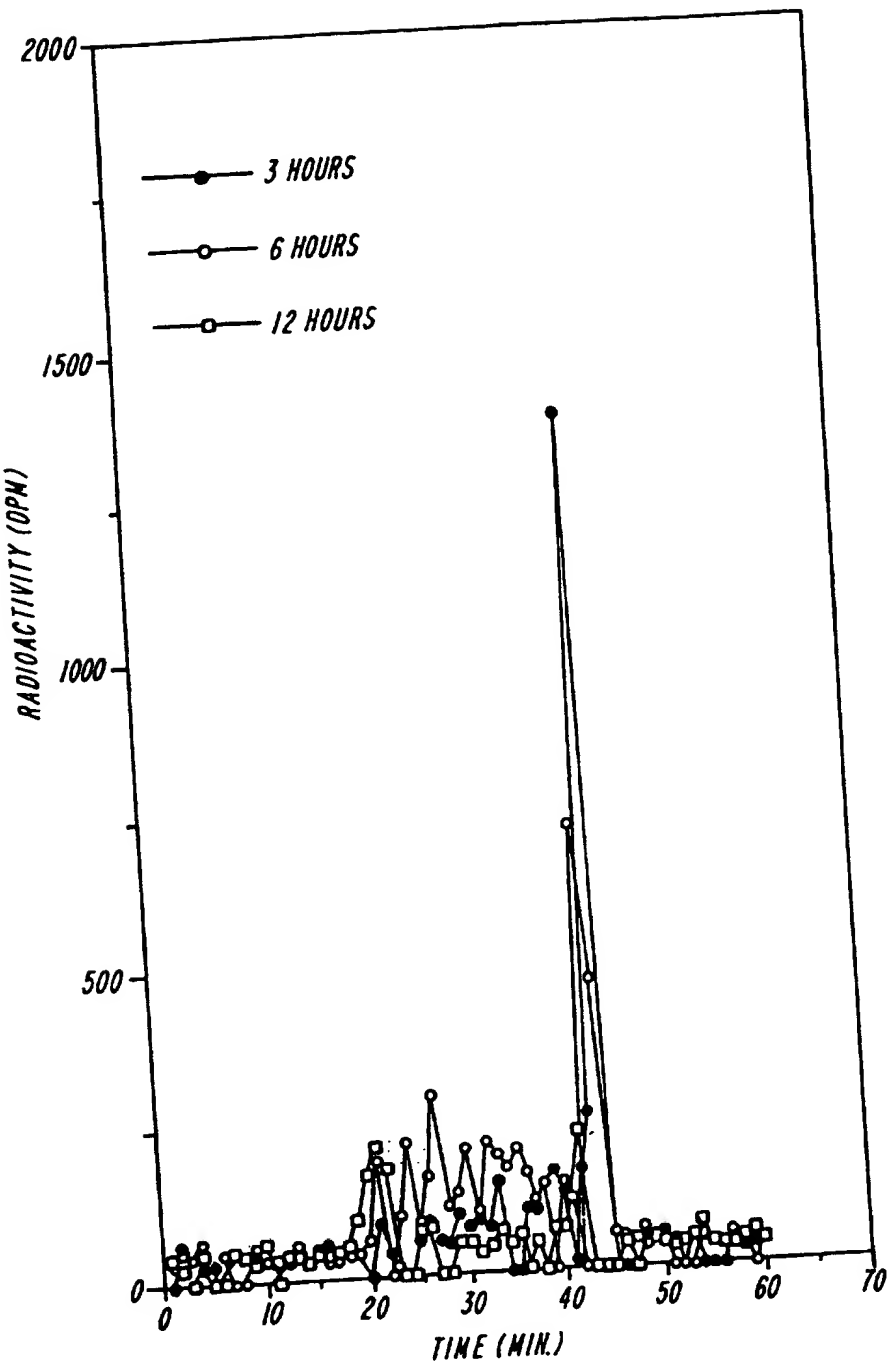
2000
1500
1000
500
0
3 HOURS
6 HOURS
12 HOURS
RADIOACTIVITY (DPM)
0
10
20
30
40
50
60
70
TIME (MIN.)

FIG. 8

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 95/13069

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 02498 (WORCESTER FOUNDATION) 3 February 1994 see page 11; table II see page 17 ---	1,3-12, 14
A	CHEM. REV., vol. 90, no. 4, 1990 pages 563-584, XP 000141412 E. UHLMANN ET AL. 'Antisense oligonucleotides: a new therapeutic principle.' --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
28 February 1996	12.03.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Klaver, T

Form PCT/ISA/210 (second sheet) (July 1992)

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. MED. CHEM., vol. 36, no. 7, 1993 pages 831-841, XP 000562792 A.M. KAWASAKI ET AL. 'Uniformly modified 2'-deoxy-2'-fluoro phosphorothioate oligonucleotides as nuclease resistant antisense compounds with affinity and specificity for RNA targets.' ---	
A	PROC. NATL. ACAD. SCI, vol. 86, no. 20, 1989 pages 7790-7794, XP 000070876 S. AGRAWAL ET AL. 'Inhibition of human immunodeficiency virus in early infected and chronically infected cells by antisense oligodeoxynucleotides and their phosphorothioate analogues.' cited in the application -----	

1

INTERNATIONAL SEARCH REPORT

.rnational application No.
PCT/US95/13069

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although the claims are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte onal Application No
PCT/US 95/13069

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9402498	03-02-94	AU-B-	4780193	14-02-94
		EP-A-	0650493	03-05-95
		FI-A-	950254	20-01-95
		NO-A-	950201	19-01-95
		PL-A-	307232	15-05-95